REMARKS

Claims 1, 4, and 40 have been amended and claims 41 and 42 have been added. More specifically, claim 1 has been amended to recite a "nucleic acid that encodes a polypeptide having the sequence of SEQ ID NO:7." Support for this amendment is found in original claim 1, and in the specification at, for example, paragraph 43. *See In re Gardner*, 177 USPQ 396, 397 (CCPA 1973) and MPEP §§ 608.01(o) and (l).

Claim 4 has been amended to recite that the isolated nucleic acid "encodes a mutant chloride intracellular channel protein whose amino acid sequence is identical to SEQ ID NO:7, except for the presence of one mutation or polymorphism." Support for this amendment is found in original claim 4, and in the specification at, for example, paragraph 36. *Id.*

Claim 40 has been amended to recite that the isolated nucleic acid "encodes a mutant polypeptide selected from the group consisting of rh133, n561 and n2400." Support for this amendment is found in original claim 40, and in the specification at, for example, paragraph 36 and Figure 5. *Id.*

New claims 41-42 have been added. Support for claims 41-42 is found in original claim 1, and in the specification at, for example, paragraphs 34-35. *Id.*

§112, Second Paragraph Rejection

Claim 40 was rejected under 35 USC §112, second paragraph. (Paper No. 20050914 at 3). In making the rejection, the Examiner asserted that "the claim encompasses an uncertain number of variants that may bear little resemblance to the

reference sequences" and that "the metes and bounds of the claim cannot be determined." *Id.*

With a view toward furthering prosecution, claim 40 has been amended to recite that the isolated nucleic acid "encodes a mutant polypeptide selected from the group consisting of rh133, n561 and n2400." In light of this amendment, the boundary of the claim is clearly ascertainable. Nothing further is required. *See, e.g., Ex parte Wu*, 10 USPQ2d 2031, 2032-33 (BPAI 1989).

In view of the foregoing, it is respectfully submitted that the rejection has been rendered moot, and should be withdrawn.

§112, First Paragraph Rejection - Written Description

Claims 1-10 and 40 have been rejected under 35 USC §112, first paragraph. (Paper No. 20050914 at 3-4). In making the rejection, the Examiner asserted that claim 1 covers a nucleic acid that has "as few as 2 consecutive nucleotides complementary to SEQ ID NO:1." *Id.* at 4. The Examiner further asserted that "the only factor present in the claim is a partial structure in the form of an indefinite degree of complementarity to a reference sequence. There is not even identification of any particular portion of the structure that must be conserved." *Id.* The Examiner then concluded that "the specification does not provide adequate written description of the claimed genus." *Id.*

With a view toward furthering prosecution, claim 1 has been amended to remove the recitation of "a sequence complementary to SEQ ID NO:1." Accordingly, it

is respectfully submitted that the rejection of claim 1 (and claims 2, 3, 5, 7, and 9, which depend therefrom) has been rendered moot, and should be withdrawn.

We note, however, that claim 42 has been added, which recites "an isolated nucleic acid sequence that is complementary to SEQ ID NO:1 or to a polynucleotide sequence that encodes SEQ ID NO:7." Such a claim is fully described in the specification. *See, e.g.*, original claim 1 and paragraph 35 ("Accordingly, the present invention provides ... an isolated nucleic acid having the sequence complementary to SEQ ID NO:1."). Obviously, the full structure of SEQ ID NO:1 is described in the specification. Accordingly, the full structure of the complement of SEQ ID NO:1 is also described. Nothing further is required by statute or precedent. Thus, it is respectfully submitted that claim 42 is sufficiently described and is allowable.

With respect to claim 2, the Examiner asserted that "there is no identification of any portion of SEQ ID NO:1 or its complements that should be incorporated into a PNA. As noted above, claim 1 has been amended to remove the recitation of "complementary" DNA. Thus, to the extent that the rejection of claim 2 relies on the recitation in claim 1 of "complements" to SEQ ID NO:1, the rejection has been rendered moot and should be withdrawn.

We further note that PNA - peptide nucleic acid - is an artificial polynucleotide that is characterized by a neutral backbone. Indeed, as is well known, the PNA monomer is typically a 2-aminoethyl glycine linked by a methylenecarbonyl linkage to one of the four bases (adenine, guanine, thymine, or cytosine) found in DNA. We also note that the Examiner has not alleged that synthesis of a PNA is outside the knowledge of one skilled in the art. Indeed, knowledge of how to synthesize PNA has

been in the public domain since at least as early as 1991 - twelve years before the present application was filed. *See, e.g.*, Nielsen *et al.*, *Science* 254:1497-1500 (1991) (copy attached as Exhibit 1). Thus, when the template of bases is known as here (SEQ ID NO:1 or a nucleic acid that encodes SEQ ID NO:7 as recited in amended claim 1), so too is possession of a PNA having the template bases. For this reason also, the rejection of claim 2 should be withdrawn.

In making the rejection with respect to claims 4, 6, 8, 10, and 40 the Examiner asserted that "it is not known how large the genus of *all* missense mutations, nonsense mutations, point mutations, substitutions, deletions, insertions, polymorphisms, or rearrangements of the sequence of SEQ ID NO:1 or any sequence of the rh133, n561 and n2400 mutant alleles (claim 40) that could encode a channel is." (Paper No. 20050914 at 5). The Examiner admitted, however, that "isolated polynucleotides comprising the nucleic acid sequence set forth in SEQ ID NO:1, and the specific variants that define the rh133, n561, and n2400 mutant alleles ... meet the written description provision of 35 USC §112, first paragraph." *Id.* at 6.

With a view toward furthering prosecution, claim 40 has been amended in accordance with the Examiner's admission to recite an "isolated nucleic acid of claim 4, which encodes a mutant polypeptide selected from the group consisting of rh133, n561 and n2400." In view of the foregoing, it is respectfully submitted that the rejection with respect to claim 40 has been rendered moot and should be withdrawn.

With a view toward furthering prosecution, claim 4 also has been amended to recite "an isolated nucleic acid which encodes a mutant chloride intracellular channel protein whose amino acid sequence is identical to SEQ ID NO:7,

except for the presence of one mutation or polymorphism." It is respectfully submitted that such a claim is fully described by the specification, including the figures, sequence listing, examples, and original claims.

Initially, we note that support for claim 4 is found in the specification as filed. For example, paragraph 36 states

> [t]he mutated nucleic acid may contain ***one*** or more deletions, insertions, missense, nonsense, point, polymorphism ... where the mutated forms of the nucleic acids encode for a mutant chloride intracellular channel protein. Specifically, the present invention discloses an isolated nucleic acid encoding a mutant EXC-4 protein, wherein the isolated nucleic acid has a sequence identical to the sequence of SEQ ID NO:1 except for the presence of ***one*** or more ... mutations [or] polymorphisms ....

The specification also exemplifies at least three such EXC-4 mutants - rh133, n561, and n2400 - having "one mutation or polymorphism" as recited in amended claim 4. See *Id.* and Figure 5B. The specification, at paragraph 37, also provides a description of how to make such mutants:

> In addition, the mutated nucleic acid sequences of the exc-4 gene can be made using standard mutagenesis techniques, including, but not limited to, chemical-induced, linker-induced, error-prone PCR induced, or radiation-induced random mutagenesis, or chemical, oligo-directed, or PCR-based site-directed mutagenesis.

The specification, at paragraph 54, also exemplifies how to identify such mutants:

> As used herein, an "excretory cell phenotype characteristic of an exc-4 *C. elegans* mutant" is defined as an excretory cell comprising a widened lumen, a truncated excretory canal ending well short of the wild-type excretory canal

> phenotype, and an altered apical surface characterized by a set of large, closely packed cystic enlargements.

And, the specification at paragraph 66 and Figs. 14A and B, discloses the morphological phenotype of EXC-4 mutant animals:

> in exc-4 mutant animals, the topology of the apical surface is altered from a single, long, narrow tube to a set of large, closely packed cystic enlargements, some of which may be disconnected spheres (FIGS. 14A and 14B).

In sum, the specification not only describes a family of EXC-4 mutants having a single mutation or polymorphism, it exemplifies three such mutants, discloses how to make the claimed mutants, discloses how to identify the claimed mutants, and discloses the morphological phenotype for the claimed mutants. Given such a detailed description, it is respectfully submitted that claim 4, as amended, is fully in compliance with the written description provisions of 35 USC §112, first paragraph. Accordingly, withdrawal of the rejection is requested.

§112, First Paragraph Rejection - Enablement

Claims 4, 6, 8, and 10 have been rejected under 35 USC §112, first paragraph. (Paper No. 20050914 at 2-3). In making the rejection, the Examiner admitted that claims 4, 6, 8, and 10 are enabled "for a nucleic acid having the sequence of SEQ ID NO:1 and the rh133, n561, and n2400 mutant alleles thereof, recombinant vectors, cells, and methods of making the encoded protein." *Id.* at 2. The Examiner asserted, however, that "the set of *all* missense mutations, nonsense mutations, point mutations, substitutions, deletions, insertions, polymorphisms, or rearrangements of the

sequence of SEQ ID NO:1 is very large and would be expected to include a large number of non-functional embodiments." *Id.* at 2-3. (emphasis added). The Examiner then concluded that "[t]esting each one for the described activity would require undue experimentation." *Id.* at 3.

Initially, we note that it is the Examiner's burden to demonstrate that a specification is not sufficiently enabling. *In re Marzocchi*, 169 USPQ 367, 369 (CCPA 1971). To carry his burden, the Examiner must identify and clearly articulate the factual bases and supporting evidence that allegedly establish that undue experimentation would be required to carry out the claimed invention. *Id.* at 370. We further note that it is well established that claims must be separately analyzed. *Ex parte Jochim*, 11 USPQ2d 561 (BPAI 1988).

Here, the Examiner has not referred to any specific feature of any specific claim that is insufficiently enabled. To the contrary, the Examiner has simply posited, in conclusory fashion, that testing each mutant "for the described activity would require undue experimentation." (Paper No. 20050914 at 2-3).

Notwithstanding the foregoing and in an effort to further prosecution, claim 4 has been amended to recite that the isolated nucleic acid "encodes a mutant chloride intracellular channel protein whose amino acid sequence is identical to SEQ ID NO:7, except for the presence of *one* mutation or polymorphism." Thus, claim 4 (and claims 6, 8, and 10, which depend therefrom) no longer recites "the set of *all* ... mutations [and] ... polymorphisms" that apparently troubled the Examiner.

As amended, claim 4 recites nucleic acid sequences that encode polynucleotide sequences that are "identical to SEQ ID NO:7, except for the presence of

one mutation or polymorphism." Such mutants are clearly described and exemplified in the specification. Indeed, Figure 5 exemplifies three such single mutants of SEQ ID NO:7, *rh133*, *n561*, and *n2400*. Thus, for this reason alone, the rejection has been rendered moot, and should be withdrawn.

One of the seminal cases for enablement, *In re Wands*, 8 USPQ 1400 (Fed. Cir. 1988), holds that it is ***not*** undue experimentation to screen antibodies (having specific characteristics, *i.e.*, are of the IgM class with a binding affinity constant of at least 10^{-9}M) produced by hybridomas Such a screening process routinely calls for assaying thousands, even tens of thousands, of antibodies.

Here, the amended claims, which now recite the presence of "one mutation or polymorphism" would require no more, and likely significantly less, experimentation to identify a mutant compared to the screening called for in *Wands*. Indeed, here, the specification specifically describes single mutations of exc-4, such as for example, rh133, n561, and n2400. *See*, *e.g.*, paragraph 36 and Figure 5. Moreover, the specification describes how to make such mutants. *See*, *e.g.*, paragraph 37. And, the specification describes how to screen for such mutants, *e.g.*, by looking for "an excretory cell comprising a widened lumen, a truncated excretory canal ending well short of the wild-type excretory canal phenotype, and an altered apical surface characterized by a set of large, closely packed cystic enlargement." *See*, *e.g.*, paragraph 54 and Example 2.

In view of the foregoing, it is respectfully submitted that the rejection has been rendered moot, and should be withdrawn.

Accordingly, for the reasons set forth above, entry of the amendments, withdrawal of the rejections, and allowance of the claims are respectfully requested. If the Examiner has any questions regarding this paper, please contact the undersigned.

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on December 20, 2005.

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timately, it may be possible to design reagents that have similar specificity and efficiency in an intracellular milieu.

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9. Oligonucleotides were purified on an FPLC Mono Q column (Pharmacia). Concentrations were determined by assuming that 1 optical density unit at 260 nm represents 33 μg. The oligonucleotide used in Fig. 2 had the sequence 5′-TCACGCCGGAAGTGAATTCAAACAGGGTTC-3′ (cleavage site shown in bold). The oligonucleotides used in Fig. 3 had the sequences 5′-TCATGAGTAAACCGTTCAAACTGAATTCCGCTTTA-3′ and 5′-CGAGATCGAAGAGGGCGAATTCCGCATTAA-3′. The oligonucleotides used in Fig. 4 had the sequences 5′-TAAGTGCTCAGAAAACATTTCTTGACTGAATTCAGCCAACAAAAATTTTGGGGTAGGTAG-3′ and 5′-AATGGCCAACTCTCTGAAAGTTATGATTATTGAGAATTCACACGTGAAGAAAGATGACATCTGG-3′.
10. We purified RecA protein using an *E. coli* strain and a detailed protocol provided by S. Kowalczykowski of the Northwestern University Medical School in Chicago (manuscript in preparation). The strain used was JC12772 [B. E. Uhlin and A. J. Clark, *J. Bacteriol.* 148, 386 (1981)]. The purification was based on the spermidine precipitation method [J. Griffith and C. G. Shores, *Biochemistry* 24, 158 (1985)], and used a single-stranded DNA agarose column with adenosine triphosphate (ATP) elution [M. M. Cox, K. McEntee, I. R. Lehman, *J. Biol. Chem.* 256, 4676 (1981)] and a Mono Q column to greatly reduce trace nuclease contamination. The concentration of RecA protein was measured on the basis of an extinction coefficient of $^{1\%}E_{280} = 5.9$ [N. L. Craig and J. W. Roberts, *J. Biol. Chem.* 256, 8039 (1981)].
11. Microbeads were used instead of agarose slabs because of the increased surface to volume ratio and greatly shortened diffusion times. Wild-type *E. coli* strain W3110 was obtained from the American Type Culture Collection and was grown overnight in Luria-Bertani medium to an OD at 600 nm of 5. Cells (5 ml) were pelleted (30 mg wet weight), washed once with 10 mM tris-HCl (pH 7.2), 20 mM NaCl, and 100 mM EDTA, and resuspended in 1 ml of this buffer. The suspension was brought to 65°C, and added to 1 ml of 1.6% low melting point agarose (InCert agarose, FMC Bioproducts) and 4 ml of paraffin oil at 65°C. Microbeads 25 to 100 μm in diameter were formed by vortexing the suspension as described [M. McClelland, *Methods Enzymol.* 155, 22 (1987)]. Beads were digested with lysozyme and proteinase K with the ImBed kit (New England Biolabs) following the manufacturer's directions. Other lysozyme and proteinase K preparations gave equally good results. Beads were stored at 4°C and were incubated in 50 mM EDTA for 30 min and equilibrated in 25 mM tris-acetate (pH 7.5), 4 mM magnesium acetate, 0.4 mM dithiothreitol, and 0.5 mM spermidine immediately before use. Beads containing HeLa cell DNA were prepared by washing 1×10^8 cells (150 mg wet weight) twice with phosphate-buffered isotonic saline, pH 7.4, and processed as above for the *E. coli* beads, except that the lysozyme digestion step was omitted.
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13. Yields were calculated from densitometry of negatives of the photographs of the ethidium bromide stained gel (Fig. 2) or autoradiograms (Fig. 3), or with a Molecular Dynamics PhosphorImager (Fig. 4). Quantitation was checked at different exposures for the autoradiograms. Reproducibility from experiment to experiment was about 10%.
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20. Sss I, Alu I, and Hha I methylases (New England Biolabs) were active under conditions optimal for the RecA protein.
21. We have confirmed the observation that this buffer is excellent for inhibiting nonspecific or star activity of Eco RI on agarose-embedded DNA [W. W. Wilson and R. M. Hoffman, *Anal. Biochem.* 191, 370 (1990)].

22. We thank G. Aurbach, G. Felsenfeld, P. Hsieh, H. Nash, and R. Proia of NIH for reading the manuscript before submission, G. Poy for oligonucleotide syntheses, and L. Robinson for her assistance. This paper is dedicated to Gerald D. Aurbach.

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Sequence-Selective Recognition of DNA by Strand Displacement with a Thymine-Substituted Polyamide

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A polyamide nucleic acid (PNA) was designed by detaching the deoxyribose phosphate backbone of DNA in a computer model and replacing it with an achiral polyamide backbone. On the basis of this model, oligomers consisting of thymine-linked aminoethylglycyl units were prepared. These oligomers recognize their complementary target in double-stranded DNA by strand displacement. The displacement is made possible by the extraordinarily high stability of the PNA-DNA hybrids. The results show that the backbone of DNA can be replaced by a polyamide, with the resulting oligomer retaining base-specific hybridization.

REAGENTS THAT BIND SEQUENCE specifically to double-stranded DNA are of major interest in molecular biology and could form the basis for gene-targeted drugs (*1*). Sequence-specific binding to operator DNA regions is the basis for the biological function of a large number of gene-regulatory proteins (*2*). Synthetic peptides that contain the approximately 50 amino acid residues constituting the DNA binding domain of such regulatory proteins can retain the DNA binding specificity of the parent protein (*3*), but at present it is not possible to design peptides that bind to desired DNA sequences. However, pyrimidine or purine oligonucleotides bind sequence specifically to homopurine regions of double-stranded DNA by triple helix formation through T·A·T and C^+·G·C or G·G·C and A·A·T triplets (*4*). The triple-helix principle has generally been applied to homopurine DNA targets. Furthermore, oligonucleotides are difficult to prepare in large scale (millimole to mole quantities), and introduction of modified nucleobases and conjugation to other ligands present major obstacles. One way to overcome these drawbacks would be to replace the deoxyribose phosphate backbone of DNA with a polyamide backbone that was homomorphous to DNA in terms of the number of backbone bonds and the distance between backbone and nucleobase.

We wanted to design a polyamide that could recognize double-stranded DNA through Hoogsteen-like base pairing in the major groove by nucleobases or other ligands having the proper hydrogen donor-acceptor properties. Thymine was initially chosen because it can participate in stable Hoogsteen triple helices with oligonucleotides (*4*) and because it presented the fewest synthetic obstacles. The proper distances in the backbone were estimated with a computer model by constructing a normal T·A·T triplex, removing the deoxyribose-phosphate backbone of the third (the T) strand, and building a polyamide backbone in its place. Units of 2-aminoethylglycine were found to fit when the thymine was attached through a methylenecarbonyl group (Fig. 1).

The resulting polyamide nucleic acid (designated PNA-1, Fig. 1) was equipped with a helix-threading acridine (*5*) for two pur-

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poses. The DNA-intercalating acridine ligand was expected to increase the affinity for double-stranded DNA as demonstrated for analogous oligonucleotide conjugates (*6*), and the nitrobenzamido ligand of the acridine was expected to make it possible to study the DNA binding by affinity photocleavage (*7*). Furthermore, the helix-threading design of the acridine was expected to place the polyamide moiety in the major groove and the nitrobenzamido ligand in the minor groove of the DNA helix when binding to double-stranded DNA. The lysine ligand was included to give some electrostatic attraction and to increase aqueous solubility.

The interaction of PNA-1 with oligonucleotides was assayed in two ways. Binding to a complementary dA_{10} sequence was demonstrated by gel retardation (Fig. 2A), which also illustrates lack of binding to the noncomplementary dT_{10} sequence. The results also show that PNA-1 is able to displace the oligonucleotide T strand in a twofold excess of double-stranded oligonucleotides, indicating that the PNA-DNA hybrid is more stable than normal double-stranded B-DNA. The PNA-DNA affinity is so high that the duplex is reformed after denaturation in 80% formamide (Fig. 2B). With conditions under which a normal dA_{10}-dT_{10} hybrid melted at 23°C (*8*), the melting temperature T_m (temperature at which 50% of double-stranded DNA is denatured) of the hybrid between dA_{10} and PNA-1 was 86°C and that between dA_{10} and PNA-2 (Fig. 1) was 73°C.

Binding of PNA-1 to double-stranded DNA was studied with a ^{32}P-end-labeled DNA fragment containing the dA_{10}-dT_{10} target sequence (*9*). After irradiation and subsequent piperidine treatment, preferential DNA-nicking was observed in the dA_{10} sequence, with the highest efficiency at A1 (numbered from the 5′ end) and at T2 of the complementary strand (Fig. 3, A and B, and Fig. 4). These nicking results are consistent with the binding of PNA-1 along the dA_{10}-dT_{10} tract that is preferentially oriented with the acridine ligand at the 5′ end of the dA_{10}. However, the minor cleavage at A13 and T10 indicates that binding also occurs with the opposite orientation.

Footprinting experiments were conducted to support the sequence-preferential binding indicated by the photo-nicking experiments. Oligo dA-dT tracts are poor substrates for both deoxyribonuclease I and the chemical footprinting reagent methidium propyl EDTA·Fe(II); therefore staphylococcus nuclease and a photo-nicking diazo-linked acridine derivative (*10*) were used. Photofootprinting of the PNA-DNA complex with the diazo-linked acridine showed significant protection of the A_{10} target sequence (Fig. 3A), whereas no protection was observed on the T strand. Contrary to these results, increased cleavage of the T strand of the target sequence by staphylococcus nuclease was seen in the presence of PNA-1 (Fig. 3B), whereas there was no increased cleavage of the A strand. Staphylococcus nuclease cleaves single-stranded DNA [at least in DNA loops (*11*)] in preference to double-stranded DNA. Thus, this result and the very strong binding of PNA-1 to dA_{10} (Fig. 2) surprisingly indicate that the binding of PNA-1 to a double-stranded DNA target results in binding (presumably by Watson-Crick hydrogen bonding) to the complementary strand (the A strand) and displacement of the noncomplementary strand (the T strand). This displacement should render the T strand sensitive to di-

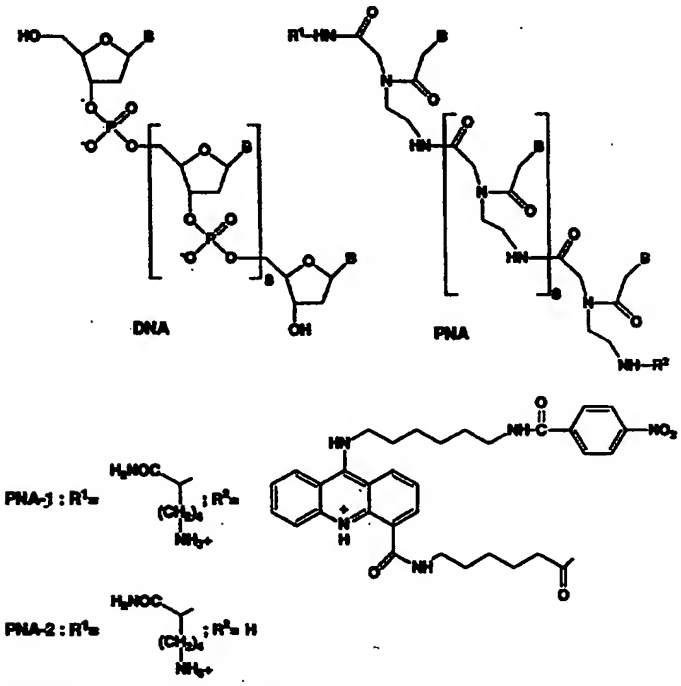

Fig. 1. Chemical structures of PNA-1 and PNA-2 (B = thyminyl) (*17*). The structure of DNA is shown for comparison.

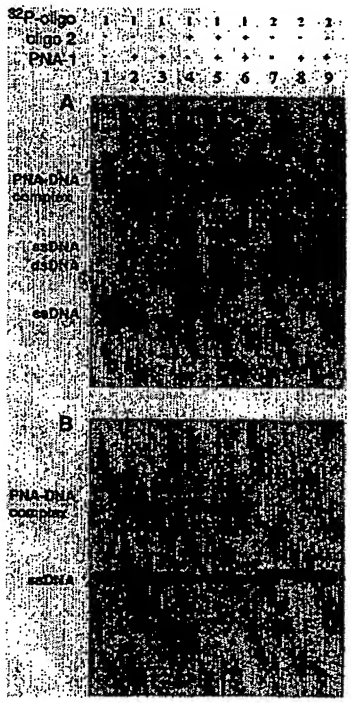

Fig. 2. Binding of PNA-1 to dA_{10} (ssDNA, single-stranded DNA; dsDNA, double-stranded DNA). 5′-^{32}P-labeled oligonucleotide 1 (5′-GATCCA_{10}G) (*16*) (lanes 1 to 6) was incubated in the absence (lanes 1 and 4) or presence of PNA-1 (lanes 2 and 5, 25 pmol; lanes 3 and 6, 75 pmol), and in the absence (lanes 1 to 3) or presence (lanes 4 to 6) of oligonucleotide 2 (5′-GATCCT_{10}G). 5′-^{32}P-labeled oligonucleotide 2 (lanes 7 to 9) was incubated in the absence (lane 7) or in the presence of PNA-1 (lane 8, 25 pmol; lane 9, 75 pmol). The samples were analyzed by PAGE and autoradiography under native conditions (**A**) or denaturing conditions (**B**). The presence of PNA-1 and oligo 2 is indicated by (+); absence is indicated by (−).

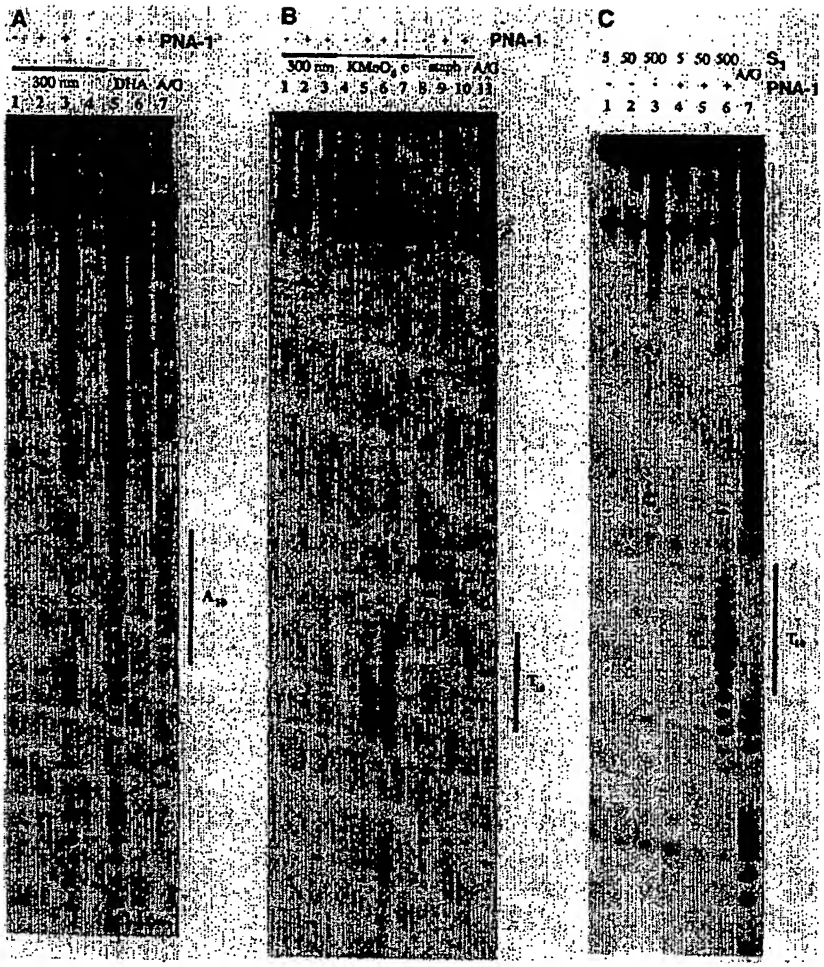

Fig. 3. Chemical, photochemical, and enzymatic probing of the dsDNA–PNA-1 complex. Either the A strand (A) or the T strand (B and C) was probed. Complexes between PNA-1 and a ^{32}P-end-labeled DNA fragment containing a dA_{10}-dT_{10} target sequence (*9*) were probed by affinity photocleavage (A and B, lanes 1 to 3; 0, 40, and 120 pmol of PNA-1, respectively); photofootprinting (A, lanes 5 and 6, 0 or 120 pmol of PNA-1, respectively); potassium permanganate probing (B, lanes 4 to 6, 0, 40, and 120 pmol of PNA-1, respectively); or probing by staphylococcus nuclease (B, lanes 8 to 10, 0, 40, or 120 pmol of PNA-1, respectively) or by nuclease S_1 (C, lanes 1 to 3, no reagent; lanes 4 to 6, 120 pmol of PNA-1; lanes 1 and 4, 0.005 U/ml S_1 concentration, shown at 1000× in figure; lanes 2 and 5, 0.05 U/ml; lanes 3 and 6, 0.5 U/ml). For lane 4 in (A), the photocleavage was performed with the free acridine carboxylate alone. In (B), lane 7 served as no-treatment control. The A+G sequence reactions are shown in (A) lane 7, (B) lane 11, and (C) lane 7.

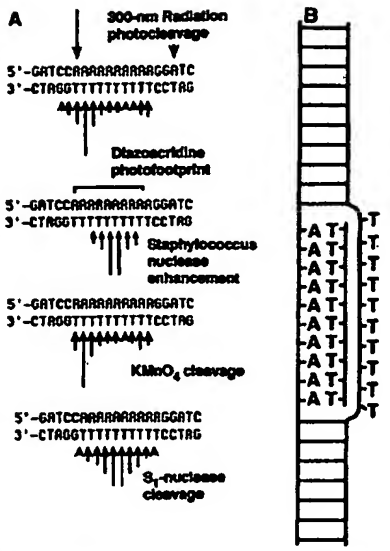

Fig. 4. (A) Schematic representation of the probing results. The length of the arrows signifies cleavage intensity. The quantitation was performed by densitometric scanning of the autoradiograms in Fig. 3 and by subtracting the corresponding background controls. For 300-nm photocleavage, lanes 2 in (A) and 2 in (B) were used with lanes 1 in (A) and 1 in (B) as background. Lanes 5 in (A) and 6 in (B) were used for DNA photofootprinting. Lane 5 in (B), with lane 4 in (B) as background, was used for $KMnO_4$ enhancement. Lane 9 in (B), with lane 8 in (B) as background, was used for staphylococcus nuclease enhancement, and lane 5 in (C), with lane 2 in (C) as background, was used for nuclease S_1 enhancement. The bracket indicates the region protected from photocleavage by the diazo-linked–acridine. (B) Cartoon of the PNA-1–ds-DNA strand-displacement complex.

gestion with single strand–specific nuclease S_1, and the thymines of this strand should be susceptible to oxidation by potassium permanganate.

Indeed, after binding of PNA-1, all thymines of the target sequence could be oxidized by potassium permanganate (Fig. 3B), and the noncomplementary strand of the target sequence was specifically attacked by nuclease S_1 and showed a symmetrical distribution of the band intensities centered at T5 (Fig. 3C), whereas no increased cleavage of the complementary A strand was seen. These observations are consistent with the proposed strand-displacement binding mode, and we are not aware of any other binding mechanism that would account for them. The acridine ligand is suspendible, but the T_m values show that it increases PNA-DNA stability (*12*). Furthermore, the observed preferred polarity of the binding may be due to the presence of the acridine.

Although strand displacement must be thermodynamically favored because of the higher stability of the PNA-DNA hybrid compared to normal double-stranded DNA, it is surprising that it takes place so readily. We believe that the acridine ligand and the positively charged lysine increase nonspecific DNA affinity and ensure a high local concentration of the PNA close to the DNA. Thus, strand displacement can be initiated through inherent DNA breathing (*13*) and proceed in a zipperlike fashion. This mechanism is supported by the observation that strand-displacement binding of PNA-1 to the target sequence, as probed by potassium permanganate hyperreactivity, is a slow process in which maximum reactivity is only observed after more than 20 min.

We believe that the high stability of the PNA-DNA hybrids is due to the lack of electrostatic repulsion between the two strands combined with the constrained flexibility of the polyamide backbone of the PNA. Although this backbone has a limited number of energetically favorable conformations because of the presence of planar amido groups, it has high flexibility at the aminoethyl linkers.

These results should apply to mixed sequences with the other three DNA bases,

and thus they may present a novel strategy to target double-stranded DNA to achieve gene modulation and to construct artificial restriction enzymes. Strand displacement may be a general principle yet to be demonstrated for other oligonucleotide analogs with a neutral backbone, and such complexes may serve as valuable models in studies of the DNA structure of transcription complexes in which strand displacement by the nascent RNA chain is a central process. The strand displacement complexes would also be analogous to three-strand DNA complexes that can be induced by the DNA recombination protein RecA.

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8. We used a Gilford response apparatus; solutions contained 10 mM phosphate, 10 mM $MgCl_2$, and 140 mM NaCl at pH 7.4. M. Egholm, O. Buchardt, P. E. Nielsen, R. H. Berg, in preparation.
9. The plasmid, pT10, containing the dA_{10}-dT_{10} target sequence was constructed by cloning of oligonucleotides 1 and 2 into the Bam HI site of pUC19 with *Escherichia coli* strain JM 101 as host. The pT10 plasmid was cleaved with Eco RI, labeled at the Eco RI site at the 3′ or the 5′ end with standard techniques (*14*), and cleaved with Pvu II, and the 248-bp fragment containing the target sequence was isolated. Complexes for probing were prepared by mixing 100,000 cpm (~1 pmol) of ^{32}P-labeled fragment with 0.5 μg of calf thymus DNA and the desired amount of PNA-1 (diluted from a stock solution of 10 mg/ml in H_2O) in 100 μl of the desired probing buffer (see below). The mixture was incubated at 37°C for 60 min before probing. Affinity photocleavage was performed in TE buffer by irradiating the sample with 300-nm radiation (Philips TL 20 W/12 fluorescent light tube, ~24 J m^{-2} s^{-1}) for 30 min. Photofootprinting was performed in TE buffer by adding 50 ng (~100 pmol) of "diazohexyl-linked-acridine" (DHA) to the sample and irradiating for 30 min at 365 nm as described (*10*). Potassium permanganate probing was done in TE buffer as described (*15*). Staphylococcus nuclease probing was done in 25 mM tris-HCl (pH 7.4), 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$ with 750 U/ml of nuclease for 5 min at 20°C. The reaction was stopped by addition of EDTA to a concentration of 25 mM. S_1-nuclease probing was performed for 5 min at 20°C in 50 mM sodium acetate (pH 4.5), 200 mM NaCl, 0.5% glycerol, and 1 mM $ZnCl_2$ with 0.005, 0.05, or 0.5 U/ml of S_1. The reaction was stopped with EDTA as above. Samples from affinity photocleavage, photofootprinting, and permanganate probing were treated with piperidine (1 M, 90°C, 20 min) before polyacrylamide gel electrophoresis (PAGE). All of the samples were analyzed by 10% PAGE in 7 M urea and TBE buffer, and ^{32}P bands were visualized by autoradiography as below (*16*). One microgram of oligonucleotide 1 was included in the samples for analysis of the A strand before gel electrophoresis to avoid retardation of the fragments in the gel due to complexing with PNA-1.
10. DHA: 9-[[6-(2-diazacyclopentadienylcarbonyloxy)-hexyl]amino]acridine; C. Jeppesen and P. E. Nielsen, *Eur. J. Biochem.* **182**, 437 (1989).
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12. DHA, $KMnO_4$, and S_1 probing results similar to those presented in Fig. 3 were obtained when PNA-2 was used instead of PNA-1.
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16. Oligonucleotides were synthesized on a Biosearch 7500 DNA synthesizer and labeled with [γ^{32}P]ATP (adenosine triphosphate) by standard procedures (*14*). We performed experiments by mixing 3000 cpm (~10 pmol) of ^{32}P-labeled oligonucleotide with the desired amount (0 or 180 pmol) of oligonucleotide 2 in 20 μl of 10 mM tris-HCl and 1 mM EDTA at pH 7.4 (TE buffer). After incubation at 20°C for 30 min the desired amount of PNA-1 was added, and the samples were incubated for a further 30 min at 20°C and 15 min at 0°C. The samples were divided into two: (*i*) to a 10-μl aliquot was added 1 μl of glycerol and 1 μl of 10 × TBE (1 × TBE = 90 mM tris-borate, 1 mM EDTA, pH 8.3), and it was analyzed by 15% PAGE in TBE buffer at 4°C; (ii) another 10-μl aliquot was evaporated to dryness, redissolved in 10 μl of 80% formamide in TBE buffer, heated to 90°C for 5 min, and analyzed by 15% PAGE with 7 M urea. Radioactive bands were visualized by autoradiography (Agfa curix RP1 film, −80°C, exposed overnight with intensifying screens).
17. The thymine monomer was synthesized by alkylation of thymine with methyl bromoacetate, subsequent hydrolysis, and conversion to the pentafluorophenyl ester with dicyclohexylcarbodiimide (DCC) before attachment to *N*-(2-Boc-aminoethyl)-glycine (Boc = butoxycarbonyl). The Boc-protected monomer was activated by conversion to the pentafluorophenyl ester. The PNA oligomers were synthesized by standard Merrifield synthesis with the Boc-benzyl strategy on a 4-methylbenzhydrylamine resin. All couplings but one proceeded with an efficiency of ≥99%, and in a typical synthesis, 24 mg of PNA-1 (80% purity) was obtained after HF cleavage of 76 mg of PNA resin. The crude product was purified by reversed-phase high-pressure liquid chromatography (>98% pure) and characterized by plasma-desorption mass spectrometry.

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Type-Specific Regulation of Adenylyl Cyclase by G Protein βγ Subunits

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Heterotrimeric guanine nucleotide–binding regulatory proteins (G proteins) dissociate into guanosine triphosphate (GTP)–bound α subunits and a complex of β and γ subunits after interaction with receptors. The GTP-α subunit complex activates appropriate effectors, such as adenylyl cyclase, retinal phosphodiesterase, phospholipase C, and ion channels. G protein βγ subunits have been found to have regulatory effects on certain types of adenylyl cyclase. In the presence of $G_{\alpha s}$, the α subunit of the G protein that activates adenylyl cyclase, one form of adenylyl cyclase was inhibited by βγ, some forms were activated by βγ, and some forms were not affected by βγ. These interactions suggest mechanisms for communication between distinct signal-transducing pathways.

G PROTEINS ACT AS TRANSDUCERS BY coupling membrane-bound receptors to intracellular effectors. G proteins are heterotrimers and are believed to dissociate to liberate a nucleotide-bound α subunit and a complex of β and γ subunits when the proteins are activated by the binding of GTP (*1*). Functional characterization provided the first basis for classification of G proteins: G_s is the G protein that activates adenylyl cyclase, and G_t (transducin) is the retinal G protein that activates a guanosine 3′,5′-monophosphate–specific phosphodiesterase. In each of these cases, the dissociated GTP-α subunit complex activates the effector enzyme (cyclase or phosphodiesterase). Thus, the concept arose that each G protein oligomer contains a functionally specific α subunit in association with mixtures of a small number of different β and γ subunits. Nearly 20 distinct α subunits have now been described, as well as four β subunits and a similar number of γ polypeptides (*2*).

Although interest has centered on the idea that α subunits are the elements that provide specificity in G protein–mediated signal transduction systems, it was suggested that

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